EFFECT OF NITROUS OXIDE ON THE MITOSIS OF PLANT CELLS

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Abstract

I have established the dependence of the metaphase-blocking effect of N_2O on gas concentration, as well as on the length of the treating period. The highest mitotic activity can be achieved in the cells of the root meristem of rye at 6 atm pressure of N_2O (270 mM concentration) by an 8 hours treatment. The metaphase-blocking effect of N_2O is reversible, following the solution of gas treatment, the metaphase-cells enter into a more or less normal anaphase.

Introduction

In investigating the phenomena of mitosis, a very great role is played by certain cytostatic materials, which hinder the regular process of mitosis. The agents hindering mitosis are called "mitotic poisons" (BIESELE, 1958). As a result of the hindering effect, there may occur specific chromosome-configurations, or polyploidy may be formed.

The mechanism of action of some agents, e.g. colchicine — the alcaloid of *Colchicum autumnale* — and of colchicum derivatives have been studied for long decades. EIGSTI and DUSTIN (1954) established that colchicine brought to stop metaphase. Its effect is so characteristic and specific in mitosis that in literature the designation C-mitosis, colchicine-mitosis, was introduced for this phenomenon (LEVAN, 1938). Apart from colchicine, colcemide has exerted the most marked effect on mitosis (PICKETT-HEAPS, 1967; STUBBLEFIELD and BRINKLEY, 1966; BRINKLEY et al., 1967).

Certain gases (xenon, argon, methane, hydrogen, nitrous oxide) are known to make an effect on the process of mitosis that is similare to that of colchicine. The mechanism of action of these gases has been unknown, so far. The nitrous oxide has produced C-mitosis in pea seedlings under atmospheric pressure (ÖSTERGREN, 1944); on the other hand, in onion a higher pressure was to be applied in order to get similar result (FERGUSON et al., 1950). In 1968, RAO treated mammaliann (HeLa) cells with N_2O and established that the effect of N_2O blocking to metaphase depended on pressure and was reversible. A pressure below 2.72 atmospheres produced no effect on the above mentioned cells. An atmospheric pressure between 2,72–4.42 atm has not caused full inhibition. A full metaphase block could be achieved with a pressure between 5.1 and 5.4 atm and the effect proved to be reversible. The degree of reversibility depends upon the duration of treatment and pressure.

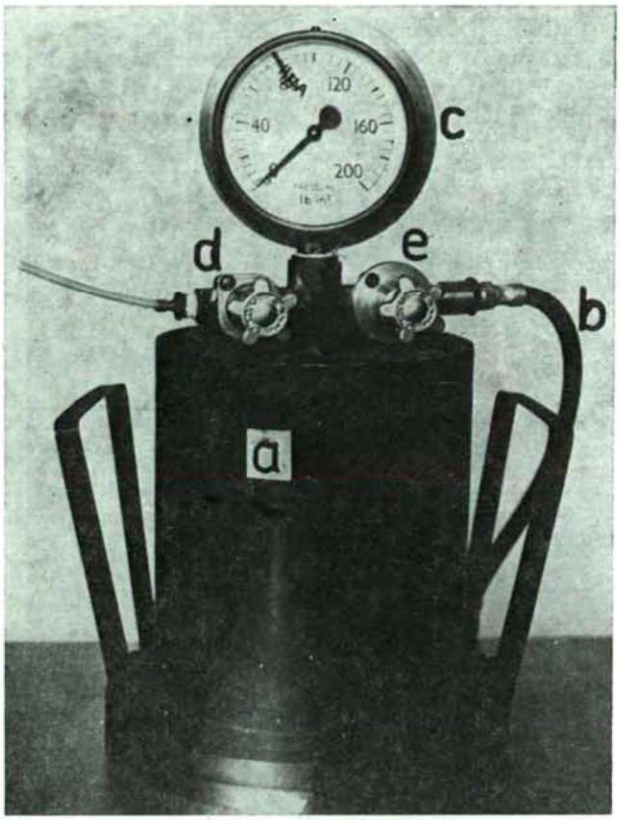

Fig. 1. Pressure chamber used for the nitrous-oxide treatment.
Pressure chamber (a), joint duct (b), manometer (c). The standard pressure in the cylinder was assured by closing both valves (d, e). Finishing the treatment, I decreased the pressure to zero atmosphere by opening the valve (d) slowly.

Similarly to colchicine and colchicine-derivatives, polyploids and aneuploids can be induced by nitrous oxide, as well (ÖSTERGREN, 1954; NYGREN, 1955; ÖSTERGREN, 1957; KIHARA and TSUNEWAKI, 1960; TSUNEWAKI, 1962, DVORAK et al., 1973).

The investigations concerning the effect of nitrous oxide are important from more than one point of view. On the one hand, gas is applied as anaesthetic in the clinical praxis, as well; on the other hand, mode of action of this, exerted on mitosis, is unelucidated, as yet.

Materials and Methods

I have chosen the diploid rye as test plant, having low chromosome number ($2n=14$). Cytological preparations were made from the actively growing root meristems of seedlings. Germination took place at 20 °C, in Petri dishes, on wet filter-paper, in a thermostat, for 20 to 30 hours. Seedlings having three roots — of 6 to 10 mm — were used for experiments. The N_2O treatments were carried out in a pressure chamber, to be seen in Fig. 1, made in the workshop of the University Cambridge. (A gift of Dr. R. T. JOHNSON to the BRC.)

The seedlings were collected for the experiment on a filter-paper in a Petri-dish and put them in a cylinder (a), treating the with N_2O of different concentrations, for a different time.

The plants were fixed in a mixture of absolute alcohol and acetic acid in ratio 3:1, at room-temperature, for 1/2 to 12 hours. The fixed seedlings were stored in 70 per cent alcohol till being used.

The 6 to 10 mm roots of the fixed seedlings were cut, then hydrolized in 1N hydrochloric acid at 60 °C for 16 to 18 minutes and stained in Schiff's aldehyde reagent according to Feulgen's squash method for 40 to 60 minutes.

The comparatively hard and thick plant cell wall renders more difficult making preparations, therefore roots were treated, before being squashed, in non-buffered enzymesolution — containing 2 per cent pectinase (FLUKA, SCHUCHARDT) and 2 per cent cellulase (ONOZUKA R-10, YAKULT) — at room-temperature. In ten minutes, from the softened roots, the enzymesolution was replaced by absolute alcohol. The roots were immersed into concentrated acetic-acid for 1 to 2 seconds and placed on a microscopic slide, and a 2 to 3 mm apical part of roots were cut. After dropping 45 per cent acetic-acid on them the root-tips were covered and squashed. The preparations of suitable quality were fixed with dry-ice method (CONGER and FAIRCHILD, 1953). After freezing, the coverslip was removed and the preparations were dehydrated in alcohol-series, then the slides were immersed into xylene. On the clean coverslips De Pex (G. T. Gurr) was dropped and then the preparations were mounted. The preparations, fixed in this way, can be stored without discolouration and pollution.

The investigations were carried out, and the needed microphotographs made, with a ZEISS NU-2 microscope, on black and white ORWO 15 Din negative film.

The mathematical evaluation of data was carried out the mean of data and the standard error of means were calculated.

The test of homogeneity of variance was carried out with Bartlett's test.

The significance test was performed with a x^2 test, the comparison of arithmetic means with a t-test (SVÁB, 1973).

The obtained results are fixed in a Table and shown in a column diagram.

Results

1) Determination of the gas concentration needed to the optimal inhibition of mitosis (Pressure experiment)

The first question to be answered was, by which nitrous-oxide pressure (concentration) the inhibition of mitosis can be achieved, in case of a treatment of indentical duration. I looked for an answer to, in which degree the mitosis of the root-meristem cells depends upon the N_2O concentration. Seedlings were treated with N_2O under a pressure of 1–12 atm, for two hours. I repeated the experiments three times, on every pressure value, i.e. I investigated 2.000 cells of the root tips of 3 times 3 seedlings in each of the experiments. It was established that, changing the nitrous--oxide concentration, the degree of mitosis-inhibition effect of gas changes, depending upon the pressure. I selected the "optimum" pressure value within the investigated interval, in case of which the highest mitotic activity can be achieved in two hours. The degree of mitotic activity i.e. the ratio of mitotic and interphase cells — considering the length of cell cycle as standard — depends on the effectivity of the inhibition of mitosis. The more effectual the inhibition is, the more mitotic cells are to be found in the seedlings simultaneously.

The inhibition of mitosis manifests itself therein that the mitosis of cells gets only as far as the metaphase. Chromosomes are arranged in a characteristic ring-form (Fig. 2) and the metaphase is not followed by ana- and telophases.

The summary of the experimental data is contained in Table 1. Beyond mitotic activity, I determined the ratio of pro-, meta-, and anaphasic cells at any pressure. The test of homogeneity of the standard deviation, Bartlett's test was used. By

Fig. 2. The "ring" metaphases, coming into existence as a result of nitrous-oxide, at the treatment at 6 atm for two hours (magnification: x1000).

means of this, I established that these do not differ significantly from each other. It was proved by comparing the means with F-test that there is a significant difference between the average mitotic activities of the seedlings, treated under different pressures, at P 1 per cent level. It was controlled with T-test, between the averages of which treatments there is a significant difference and the value of the minimal significant difference was also calculated. In my experiments, this proved to be 1.87 per cent. It is therefore due to the effect of nitrous oxide if in the treatments the mitotic activity differs from the control level by more than 1.87 per cent or if the difference of mitotic activities measured in two treatments is more than 1.87 per cent. It can be established on the basis of significance-investigations that there are significant differences between the treatments of 1 to 3, 4 to 5, 5 to 6, 6 to 7, 7 to 8, 8 to 10, 10 to 12 atmospheres. The diagram of mitotic activity — and the ratio of the

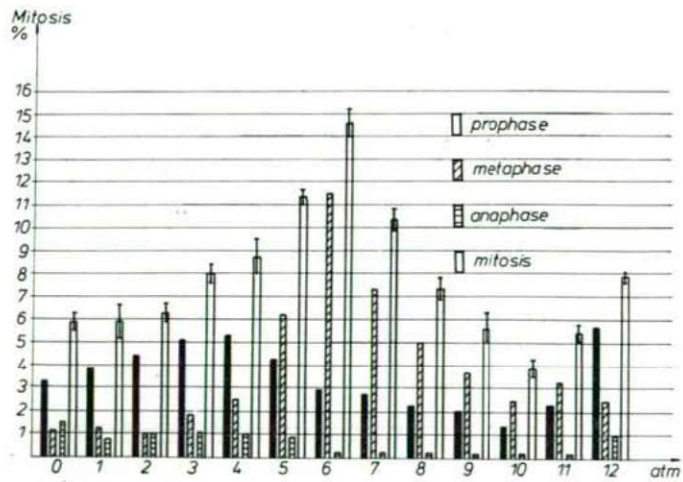

Fig. 3. The pro-, meta-, and anaphase change in the cells of the root meristem, as well as that in the mitotic activity, taken as a function of pressure. Treatment is: 2 hours.

pro-, meta-, and anaphase cells — is to be seen in Figure 3, in which the dynamics of the change in the single mitotic phases can be followed well.

It turns out from the diagram unambiguously that by the change in concentration the ratio of the cells in the mitosis also changes, as compared with the mitotic activity of control cells. A significant inhibition of mitosis (mitotic accumulation) is to be observed, begining from a gas concentration of 3 atm (135 mM). Till atm 6, the number of mitotic cells increases, after this it decreases (at atm 9 to the level of control). At 10 atm, there are fewer cells in mitosis than in the root-tip of the control. This little but significant decrease in the number of mitotic cells is due to the vigorous decrease in the number of prophase cells. At this pressure, the number of prophase cells does not achieve the level of 50 per cent of the control. As this trend ceases to continue after the increase of pressure, and at 12 atm the number of mitotic cells considerably exceeds even the level of control, the effect of the 10 atm pressure cannot be considered as a "saturation".

Analysing the distribution of the phases of mitosis (pro-, meta-, and anaphases), we can conclude that with changing of the gas pressure, the most obvious change follows in the number of metaphases. At 6 atm, the number of metaphase cell increases almost to be 12-fold, as compared with the control. The number of pro phases remains, apart from the decrease mentioned above, approximately on an identical level. Following the formation of anaphases with attention, I have found that the ratio of anaphases shows till a pressure of 5 atm (225 mM gas concentration) a value close to the control. It is, between 6 to 11 atm not more than 10 to 15 per cent of the control, showing unequivocally the full blocking of the metaphase. The final conclusion from the pressure experiment that the highest mitosis accumulation can be achieved at a pressure of 6 atmospheres.

The question arose whether the metaphase-blocking effect of nitrous oxide depends, and in what degree, on the length of the time of treatment.

2) The effect of duration of treatment on the mitotic accumulation (Time experiments)

Seedlings were treated at a pressure of 6 atm and two seedlings were fixed hourly, resp. two-hourly, each, for 1 to 34 hours. The percentage of mitotic, pro-, meta-, and anaphase cells was calculated from a thousand cells, each. The experiment was repeated three-times. The data are summarized in a Table (Table 2).

With Bartlett's test, the investigation the test of homogeneity of the variance were performed. On the basis of mathematical evaluation, the standard deviation of the data of the 20 experiments does not differ significantly from one another. The comparison of arithmetic means was performed with F-test. It is to be established on the basis of the F-test that between the averages of the different treatments there is a obvious significant difference. The single treatments were controlled with t-test in detail and I exactly established between the averages of which groups least significant differences are. I calculated the least significant difference, this proved to be 2.55 per cent. The dependence of the pro-, meta-, and anaphases and of mitotic activities on the time is plotted on column diagram (Fig. 4).

On the basis of analysing the data, it is to be established that the mitotic activity depends on the duration of treatment. The highest mitotic activity was faund at 8 hours treatment (Fig. 5). A treatment longer than 8 hours gradually decreases

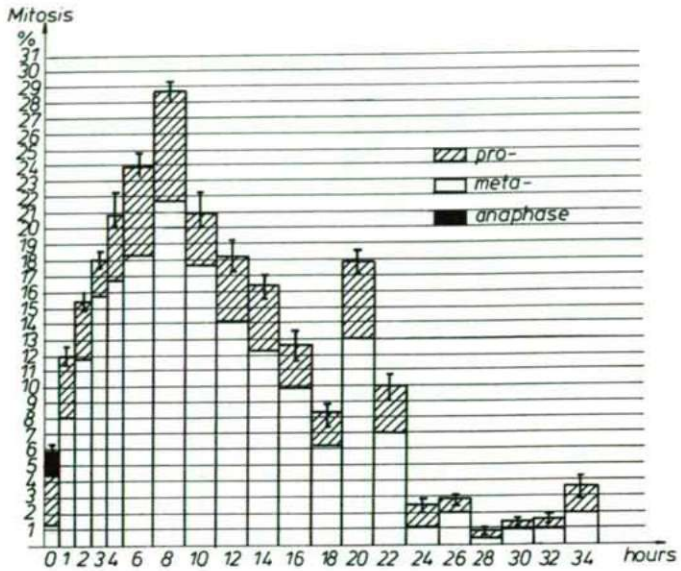

Fig. 4. Change in the mitotic activity of the root-meristem cells, taken as a function of the treatment of time, as a result of a 6 atm gas treatment.

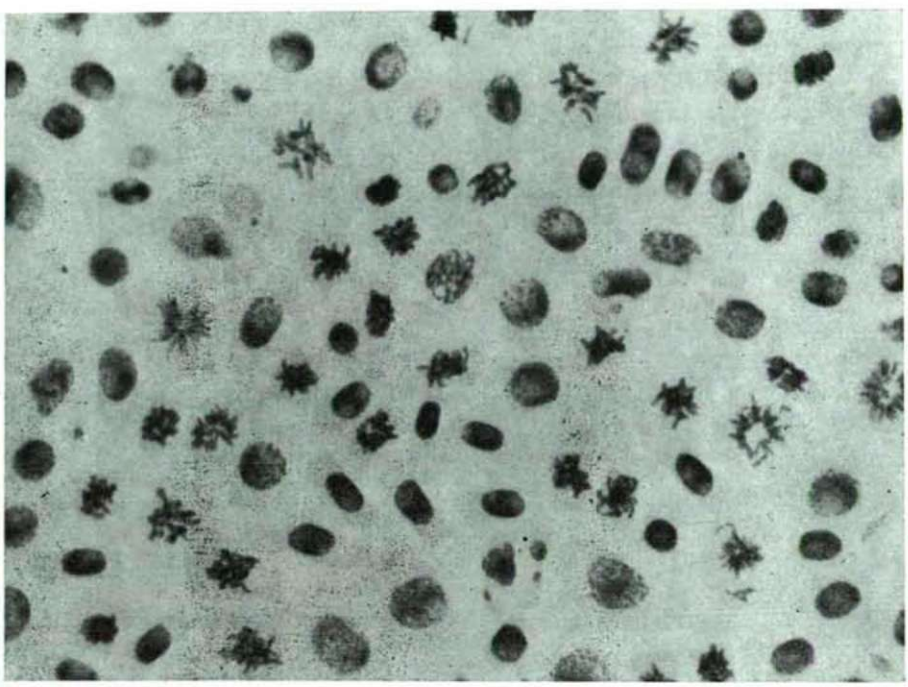

Fig. 5. The effect of an 8-hour nitrous-oxide treatment, at 6 atm pressure, on the division of cells. Magnification: x400.

the number of the cells in mitosis, while the curve reaches its first minimum at 18-hour. It is obvious that this minimum is still higher than that to be observed in the control. At a 20-hour treatment, again a sharp peak can be observed on the curve. The majority of cells at that point are tetraploid. Following this, the mitotic activity begins to decrease steeply till the 20-hour treatment, then it remains below the level of the control all the while.

Studying the given phases of the mitosis, it was faund that after a 2-hour treatment the ratio of anaphases remained on the level of 10^{-4} frequency, i.e. it could be considered as zero. The change in the number of metaphases cells follows the change in the mitotic activity well. With the exception of treatments for 2 and 3 hours the frequency curve of the number of prophase cells is also of like course.

Knowing the nitrous-oxide concentration, blocking the most effective mitosis, as well as the optimum time of treatment, I found it necessary to perform some control experiments for proving that really the specific effect of N_2O is really in question and not the changes by the experiental conditions (high pressure). For the sake of this, at 6 atm pressure, I performed 8-hour treatments with air, nitrogen, and oxigen. At none of these I observed any cytological event, similar to those observed during of the nitrous-oxide treatments. In all the three treatments, mitotic activity remained at control level or — within the limits of significancy — below the level of the control.

3) Deciding the reversible resp. irreversible effect of the nitrous-oxide treatment (Experiment of returning)

In order to decide whether that the effect of nitrous-oxide on mitosis is a reversible process or not I have performed the following experiments. The seedlings were treated at 6 atm pressure for eight hours. Then, after solving the effect of N_2O seedling were fixed in 1/2 resp. 1 hour intervals. The last sample was taken after the termination of gas treatment, in the sample was taken after the termination of gas treatment, in the 21st hour. The experiment was repeated three times. In all the three repeated samples, the percentage of the mitotic, as well as pro-, meta-, and anaphasic cells was counted from 4000 cells, each. The data, obtained from the investigation, are showed in Table 3.

Mathematical control of data, was performed establishing that the standard deviations are not significant, the average values are to be considered as homogeneous. The least significant difference proved to be 2.93 per cent.

The averages of mitotic, pro-, meta-, and anaphasic activities are plotted, like before, in a column diagram, taken as a function of time (i.e. the time following gas solution) (Fig. 6). In the Figure 6 it is to be seen that, after solving gas pressure, mitotic activity decreases abruptly and the anaphase cells immediately appear (Fig. 7).

Following the formation of the certain phases of mitosis, we can see that in 30 minutes after solving gas pressure the number of anaphases rises from zero per cent to 7.3 per cent, then it gradually reaches the control lever. After ten hours, mitotic activity again has an abrupt peak, being almost 2.5 times more than the control value. After ten hours, mitotic activity shows a five-hour periodicity, each presenting itself in the form of a peak, essentially exceeding control level. In these

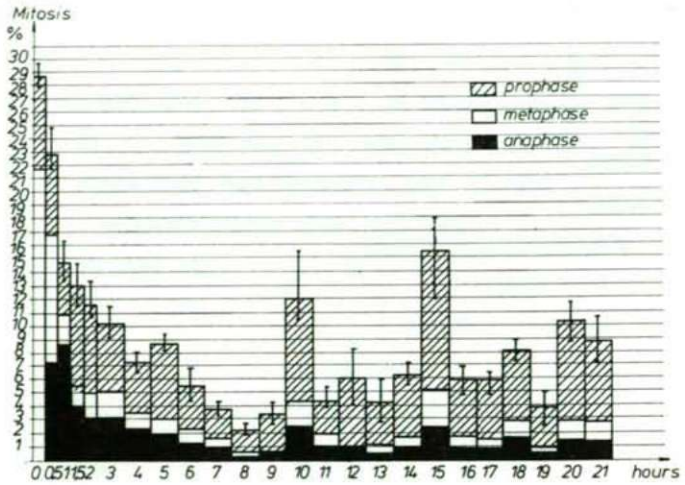

Fig. 6. Change in the pro-, meta-, and anaphase, as well as mitotic activities of the root-meristem cells, taken as a function of the time following gas solution. The treatment lasted at 6 atm pressure, for 8 hours.

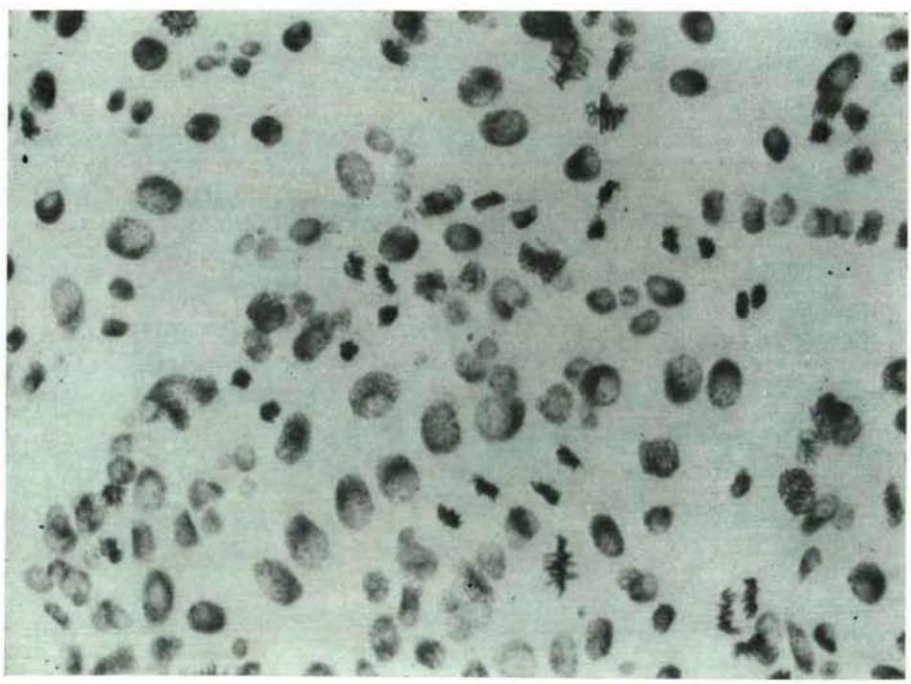

Fig. 7. Change in mitoses, at 6 atm, following the termination of the 8-hour gas treatment. Magnification: x400.

peaks, more than two-thirds of mitotic cells are formed by prophase cells. Apart from peaks, beginning from the 5th hour following gas treatment, the value of mitotic activity does not differ from the control level significantly.

Discussion

At evaluating the effect made by nitrous oxide on the dividing root-meristem of seedlings, it is absolutely necessary to discuss certain questions which are to be considered as peculiarities of the experimental system. For lack of this, the interpretation of experimental results, the conclusions drawn may be the sources of several errors.

It is to be considered as one of the most important questions that *the root-meristem cells cannot be regarded as a homogeneous cell population.* In the root tip, fast-cyclizing cells (of short cell-cycles), slowly cyclizing cells (of long cell-cycles) and non-cyclizing (resting) cells are to be found. It is not yet settled whether these resting cells are only in a provisional resting state or they remain definitely "out of the cell cycle". For the time being, there is no direct evidence for that these cells have lost their mitotic activity (CLOWES, 1976). It is obvious that the existence of these three basic cell types makes extremely complicated to evaluate any effect and this fact is absolutely to be had in sight. At elaborating the results, therefore, we should leave out of consideration every evaluation supposing a uniform cell cycle (i.e., some cells dividing with identical speed, identical rate). The root-tip preparations made in the course of investigations consisted on average number of 3500 to 4000 cells. In the certain experiments, we evaluated uniformly 2000 cells, each, which is approximately 50 per cent of the full preparation. I should have liked to decrease to minimum the errors given by the different "answers" of "fast", "slow", "non-dividing" cells.

Table 1. Determination of the gas concentration, needed to inhibit the optimum mitosis (Pressure experiment). Average values of the pro-, meta-, and anaphases, as well as of mitotic activities, and the dispersion values of the arithmetical average of mitocic activities. (The time of the nitrous-oxide treatment is two hours at each pressure.)

Mitosis N_2O pressure (atm)	Prophase	Metaphase	Anaphase	Mitosis	$s_{\bar{x}}$**
Control	3.32	1.09	1.48	5.89	0.41
1 atm	3.79	1.24	0.84	5.87	0.79
2 atm	4.40	0.96	0.98	6.34	0.43
3 atm	5.07	1.84	1.06	7.97	0.39
4 atm	5.28	2.50	0.92	8.70	0.80
5 atm	4.19	6.18	0.89	11.26	0.34
6 atm	2.93	11.35	0.23	14.51	0.57
7 atm	2.74	7.34	0.24	10.39	0.49
8 atm	2.16	4.98	0.15	7.29	0.51
9 atm	1.97	3.61	0.06	5.64	0.57
10 atm	1.32	2.39	0.12	3.83	0.39
11 atm	2.17	3.16	0.11	5.44	0.28
12 atm	5.58	2.40	0.86	8.84	0.20

** $s_{\bar{x}}$ Standard deviations of the averages of mitotic activities.

Using 2-hours experimental times during the pressure experiments (Table 1, Fig. 3), I have established that within the investigated series of 1–2 atm pressure intervals, *a mitotic accumulation* can be achieved at a pressure of 6 atmospheres. The accumulating effect of the nitrous-oxide mitosis can unambigously be explained by increasing the number of metaphases, i.e. by metaphase block of cell division. Owing to technical causes (lack of an apparatus of high pressure), I could not investigate into the repeated rise of the mitotic activity at 12 atmospheres. Thus, I cannot give any satisfactory explanation to this. After establishing the "optimum" gas concentration (6 atm), I investigated into the effect of the time of treatment on the mitotic accumulation, within an interval of 1 to 34 hours (Table 2, Fig. 4). The number of mitotic cells is roughly doubled by only 1-hour treatment at 6 atm pressure, this being the result of an about 7-fold increase in the number of metaphase cells, as compared with the control. The highest mitotic accumulation was achieved at the treatment of 8 hours, where the ratio of metaphase cells was about 20-fold, comparing with the control.

There is to be found more than one explanation for the rise in the number of prophases, namely: 1) The prophase phase relatively lengthens, while the time of cell cycle remains unchanged. 2) The cell cycle accelerates, 3) mitosis-induction in the non dividing cell population, etc. The number of metaphases gradually decreases after a treatment for eight hours, from which the conclusion can be drawn that the metaphase-blocking effect of the nitrous-oxide gas is restricted in time and, after a treatment exceeding the 8 hours, already more cells go over to an interphase,

Table 2. Effect of the time of treatment on the mitotic accumulation (Time experiment). Average values of the pro-, meta-, and anaphases, as well as of mitotic activities, and the dispersion values of the arithmetical average of mitotic activities. (The nitrous-oxide treatment took place at six atmospheric pressure.)

Mitosis Time (hrs)	Prophase	Metaphase	Anaphase	Mitosis	$s_{\bar{x}}$**
Control	3.32	1.09	1.48	5.89	0.41
1	3.53	8.10	0.05	11.68	0.63
2	3.18	11.88	0.28	15.34	0.72
3	2.35	15.67	0.09	18.11	0.40
4	4.15	16.57	0.00	20.72	1.58
6	5.42	18.23	0.12	23.77	1.03
8	6.83	21.70	0.03	28.56	0.58
10	4.78	17.63	0.03	22.44	1.47
12	4.12	14.07	0.07	18.25	0.96
14	4.08	12.23	0.00	16.31	0.68
16	2.55	9.92	0.02	12.49	1.12
18	2.00	6.25	0.00	8.25	0.53
20	4.80	12.97	0.00	17.77	0.59
22	3.03	6.98	0.00	10.01	0.83
24	1.32	1.27	0.00	2.59	0.30
26	0.80	2.08	0.00	2.88	0.33
28	0.28	0.57	0.00	0.85	0.14
30	0.60	1.00	0.00	1.60	0.22
32	0.67	1.05	0.00	1.72	0.26
34	1.72	2.10	0.00	3.82	0.59

** $s_{\bar{x}}$ Standard deviations of the averages of mitotic activities.

omitting the ana- and telophasic phases than the number of cells going newly into mitosis. After 20 hours, the number of metaphase again shows a rise. But most of the mitotic cells are after twenty hours are tetraploid. The peak is, therefore, given not by cells stepped into a "new" mitosis, but already "treated" cells get again till the metaphase. From this, the conclusion can be drawn that:

1) by nitrous oxide, no notable inhibition is induced in another stage of the cell cycle,

2) during the 6 atm nitrous-oxide treatment, the cycle time of these cells is approximately 10 hours. A treatment shows after 22 hours an anequivocal inhibitory effect and till a treatment for 34 hours I have not observed any following rise. Beyond the inhibitory effect induced by the long treatment, a part can be played in this by the exhaustion of dividing ability of the cells already divided.

With control experiments, I established that *the effect of nitrous oxide on the mitosis is specific*, and it is not due to the physical conditions of the treatment (e.g. high pressure). 6-atm, 8-hour treatments were performed with air, nitrogen, and oxygen and none of these resulted in an effect, similar to that of N_2O.

Table 3. Decision on the reversible resp. irreversible effect of the nitrous-oxide treatment. (Experiment of returning.)
Arithmetical averages and dispersion values of the pro-, meta-, and anaphases, as well as of mitotic activities.
(Nitrous-oxide treatment at 6 atm pressure, for 8 hours, sampling following gas solution, in different points of time.)

Mitosis Time (hrs. min.)	Prophase	Metaphase	Anaphase	Mitosis	$s_{\bar{x}}$ **
Control	3.32	1.09	1.48	5.89	0.41
0.00	6.84	21.70	0.03	28.60	0.58
0.30	5.80	9.51	7.28	22.78	0.99
1.00	3.85	2.22	8.60	14.67	0.75
1.30	7.33	1.53	4.13	12.97	0.83
2.00	6.48	2.13	3.00	11.60	0.94
3.00	4.90	2.10	3.17	10.17	0.55
4.00	3.68	1.32	2.30	7.30	0.32
5.00	5.17	1.17	1.86	8.72	0.33
6.00	3.13	1.07	1.30	5.50	0.59
7.00	2.18	0.72	1.03	3.93	0.24
8.00	1.57	0.53	0.22	2.32	0.18
9.00	2.28	0.60	0.58	3.47	0.44
10.00	7.10	1.90	2.47	11.97	1.72
11.00	2.47	1.02	0.92	4.40	0.45
12.00	4.15	0.93	1.05	6.13	0.98
13.00	3.02	0.88	0.40	4.30	0.92
14.00	4.63	0.87	0.78	6.28	0.51
15.00	10.22	2.83	2.35	15.40	1.68
16.00	4.12	0.97	0.72	5.80	0.46
17.00	4.33	0.83	0.67	5.83	0.29
18.00	5.07	1.33	1.63	8.03	0.43
19.00	3.08	0.38	0.38	3.85	0.59
20.00	7.43	1.50	1.28	10.22	0.65
21.00	5.87	1.28	1.43	8.58	0.92

** $s_{\bar{x}}$ Standard deviations of the averages of mitotic activities.

According to my investigations, *the effect of nitrous oxide is reversible*. Following the solution of the gas treatment, the metaphase cells immediately pass over into anaphase.

After solving the gas treatment (Table 3), the change in *mitotic activity shows a definite periodicity*. For 9 hours, mitotic activity gradually decreases, then, at 10 hours, it achieves a level doubling that of the control, and followed after 15 resp. 20 hours again by a new peak, in each case.

On the data of the time experiment, it is not probable that this phenomenon would be a consequence of a partial blocking, taking place at some point of the cell cycle. I consider as a more probable explanation that the 10-hour peak is induced by the fast cyclizing cells partially remaining in synchronized, while the 15-hour peak is formed by the cells of the cell population of a faster cell cycle, remaining in synchron. The 20-hour peak is again produced by the fast cyclizing cells of supposedly 10-hour cell cycle. On the basis of this explantion, the larger share of the nearly 30 per cent mitotic cell population of zero hour zero minute would be given by the slower cells (of 15-hour cell cycle). The sum of the 10- and 15-hour peaks agrees extremely well with the mitotic activity observed at the termination of gas treatment. By this, as well as by the comparison of the 10- and 20-hour mitotic activities, the extremely high stability of synchronity is shown.

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